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(54) Title: **Tetrazolium Salts**

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Tetrazolium Salts

Tetrazolium salts are valuable reagents for visualization of biological reduction processes. They generally are colorless compounds that are converted to strongly colored, poorly soluble substances by reduction, for example, with a reducing sugar, these substances being referred to as formazans (L. F. Fieser and M. Fieser, Org. Chemie, Verlag Chemie, 1965, page 1437). It has long been known that reduced pyridine nucleotides react with tetrazolium salts in the aforementioned manner by catalysis with N-methylphenazinium salts (PMS) or the enzyme diaphorase. A number of determination methods for reduced pyridine nucleotides are based on this reaction and therefore methods for activity determination of enzymes. For example, the activity of lactate dehydrogenase (LDH) can be determined by the fact that lactate, when catalyzed by lactate dehydrogenase, is converted with nicotinamide-adenine-dinucleotide (NAD) to pyruvate and reduced nicotinamide-adenine-dinucleotide (NADH). The formed NADH now reacts, for example, in the presence of the enzyme diaphroase with tetrazolium salts to form NAD and colored formazans, whose concentration can be determined photometrically.

Only a few tetrazolium salts have thus far been available for detection of NADH (for example, triphenyltetrazolium chloride (TTC), iodophenyl-nitrophenyl-tetrazolium salts (INT), 2,5-diphenyl-3-[4,5-dimethylthiazolyl-(2)]tetrazolium bromide (MTT), which, however, are not readily suited for quantitative determinations in a photometer, since they do not adequately satisfy the following conditions necessary for this purpose:

1.) Both the tetrazolium salt and the formazan formed during reduction must be readily soluble in aqueous solution even without addition of organic solvents or solubilizers.

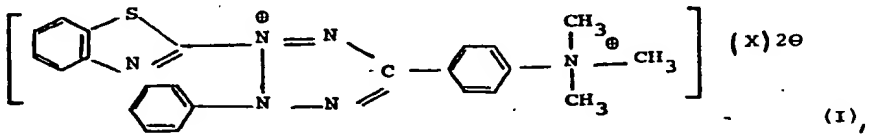
2.) The tetrazolium salt must be easily and rapidly reduced under the reaction conditions of enzymatic determination.

3.) The formed formazan must have an absorption maximum in a favorable measurement range, if possible, around 550 nm.

4.) The molar extinction coefficient and therefore sensitivity must be very high.

The purpose of the present invention was to develop tetrazolium salts that are suitable for an enzymatic NADH determination in a photometer and satisfy the aforementioned conditions in the most ideal possibly manner.

It was found that the previously unknown 2-benzothiazolyl-(2)-3-phenyl-5-(4-N-trimethylammonium-phenyl)tetrazolium salts of formula I



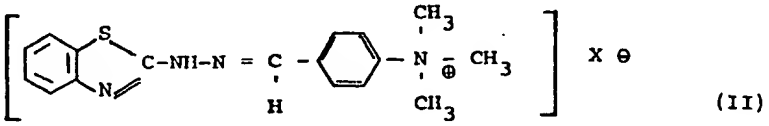
(I),

in which X denotes one or more anions of organic or inorganic acids,

are readily soluble, rapidly and sensitively react and surprisingly produce formazans that have a favorable absorption maximum at 525 nm, and are also characterized by extraordinary solubility and high molar extinction coefficient. The compounds according to the invention are therefore excellently suited for photometric determination of NADH and other reducing substances.

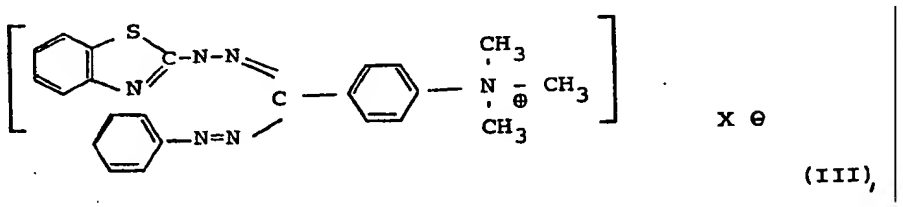
Chloride, bromide, iodide, nitrate, fluoroborate, perchlorate, sulfate, oxalate and tartrate are considered as anions. However, since the choice of anion is not of essential importance for the reactivity of the compounds according to the invention, other anions and anion combinations can be also combined with the tetrazolium cation to reactive salts. It goes without saying that anions that eliminate the sought properties of the detection reagent, for example, easy solubility, or interfere with detection, for example, by an inhibitor effect (fluorides), are not suitable.

Preparation of the substances according to the invention occurs in known fashion by conversion of a hydrazone of formula II



(II)

in which X⊖ has the aforementioned meaning, with a reactive benzene diazonium salt, preferably with benzene diazonium chloride, and then oxidation of the obtained formazan of formula III



(III),

in which X⊖ has the aforementioned meaning,

to compounds of formula I with an appropriate oxidizing agent, in which the anion or anions can be modified according to the invention by conversion with corresponding alkali salts or correspondingly charged anion exchangers.

For conversion with compounds III, the halides, preferably chlorides, are considered, in particular, as hydrazone salts II.

Mercury(II) oxide, isoamyl nitrite, lead tetraacetate, N-bromosuccinimide or N-chlorophthalimide are considered as oxidation agents for compounds III.

The compounds of formula II used as starting products are prepared in known fashion, for example, by conversion of p-N-trimethylammonium-benzaldehyde-chloride with 2-hydrazinobenzothiazole in glacial acetic acid, and optionally subsequent modification of the group X.

The tetrazolium salts according to the invention are quite generally suited for determination of reducing substances, for example, reducing sugars, ascorbic acid or ketosteroids. Only the reaction conditions need be adjusted here to the increased reactivity. The main task of the present invention, however, was to furnish a reagent for detection of biological reduction processes that is used especially to determine enzymes or their substrates, during whose reactions reduced pyridine nucleotides are formed or consumed.

Nicotine-adenine-dinucleotide (NADH), nicotine-adenine-dinucleotide-phosphate (NADPH) and acetylpyridine-dinucleotide (APADH) [sic; acetylpyridine-adenine-dinucleotide] are primarily considered as reduced pyridine nucleotides. The enzymes lactate dehydrogenase (LDH), malate dehydrogenase (MDH) and glutamate dehydrogenase (Gl. DH) can be determined according to the invention.

The following substrates can also be determined enzymatically: ethanol with alcohol dehydrogenase, glucose with hexokinase and glucose-6-phosphate-dehydrogenase, galactose with galactose dehydrogenase, as well as glycerol with glycerokinase and glycerophosphate-dehydrogenase.

Diaphorase and N-methylphenazinium-methosulfate (PMS) are suitable as reduction catalysts, in which no difference exists relative to the known tetrazolium salts with respect to experimental and reaction conditions.

The following examples serve to further explain the invention.

Example 1

Preparation of 2-benzothiazolyl-(2)-3-phenyl-5-(4-N-trimethylammoniumphenyl)tetrazolium dichloride

a) p-trimethylammonium-benzaldehyde-chloride

29.8 g (0.2 mol) p-dimethylaminobenzaldehyde is boiled under reflux with 56 g (0.4 mol) methyl iodide in 100 mL acetonitrile for 8 hours under agitation. After another two hours of agitation at room temperature, the formed colorless crystals of p-trimethylammonium-benzaldehyde-iodide are filtered by suction and washed with a small amount of ice-cold acetonitrile.

Mp. 158°, yield: 48.6 g (80.7 percent of the theoretical)

30 g p-trimethylammonium-benzaldehyde-iodide is dissolved in 100 mL water and passed over an anion exchanger (Amberlite IRA 400) loaded with chloride ions. During concentration, colorless, somewhat hydroscopic crystals of p-trimethylammonium-benzaldehyde-chloride are obtained from the eluate, Mp. 203-204°; yield: 19.8 g (= 96.3% of the theoretical).

b) p-N-trimethylammonium-benzaldehyde-benzothiazolyl-(2)-hydrazone-chloride

25 g (0.125 mol) p-N-trimethylammonium-benzaldehyde-chloride is agitated for 6 hours with 20.8 g (0.125 mol) 2-hydrazinobenzothiazole in 300 mL glacial acetic acid. After concentration in vacuum and addition of a small amount of alcohol, the substance crystallizes in light yellow crystals that are recrystallized from methanol. 39.4 g (= 90.5% of the theoretical) p-N-trimethylammonium-benzaldehyde-benzothiazolyl-(2)-hydrazone-chloride is obtained, Mp. 215°C.

c) 1-phenyl-3-(4-N-trimethylammoniumphenyl)-chloride-5-benzothiazolyl-(2)-formazan

20.7 g (0.06 mol) p-N-trimethylammonium-benzaldehyde-benzothiazol-(2)-hydrazone-chloride is dissolved in 600 mL water and 150 mL pyridine and cooled to 0°C. A benzenediazonium salt solution is then prepared from 6.5 g hydrochloric acid, 6 mL water and 2.8 mL aniline at 0°C with addition of 2.1 g sodium nitrite in 5 mL water. This

is added dropwise at 0°C during agitation and further cooling of one-half hour to the prepared hydrazone solution, whereupon the formazan forms in green crystals. After 1 hour of further agitation, the voluminous precipitate is filtered by suction and washed with about 150 mL water in small batches. After filtration, the dry residue is agitated with 200 mL dry dioxane and filtered by suction. 24 g (89.4% of the theoretical) 1-phenyl-3-(4-N-trimethylammoniumphenyl)-chloride-5-benzothiazolyl-(2)-formazan is obtained, Mp. 152°C (decomposes).

d) 2-benzothiazolyl-(2)-3-phenyl-5-(4-N-trimethylammonium-phenyl)tetrazolium-chloride

18 g (0.04 mol) 1-phenyl-3-(4-N-trimethylammoniumphenyl)-chloride-5-benzothiazolyl-(2)-formazan is dissolved in 450 mL methanol. The dark red solution is mixed with 13.5 g N-chlorophthalimide, slowly heated and boiled under reflux until a light yellow solution has formed. After cooling to 5-10°C, the excess N-chlorophthalimide (about 6 g) is filtered by suction and washed with a small amount of cold methanol. The filtrate is then mixed with 2.5 liter of ether. The separated voluminous tetrazolium salt is filtered by suction and agitated with about 250 mL ethyl acetate. The yellow crystals so obtained are recrystallized from 160 mL n-propanol for further purification, in which the dark brown solution is clarified by addition of some activated carbon. 15 g (71.3% of the theoretical) 2-benzothiazolyl-(2)-3-phenyl-5-(4-N-trimethylammoniumphenyl)tetrazolium-chloride is obtained, Mp. 156°C (decomposes).

Example 2

Preparation of further 2-benzothiazolyl-(2)-3-phenyl-5-(4-N-trimethylammoniumphenyl)tetrazolium salts

1 g of the 2-benzothiazolyl-(2)-3-phenyl-5-(4-N-trimethylammoniumphenyl)tetrazolium-dichloride, described in example 1, is dissolved in 5-10 mL water and slowly mixed under good agitation with the solid sodium salt of the desired anion. The more or less rapidly precipitating tetrazolium salts are filtered by suction and recrystallized.

The following were prepared in this manner:

Dibromide: yellow crystals from ethanol, Mp. 174-176°C (decomposes)
Diiodide: red needles from methanol, Mp. 160-163°C (decomposes)
Difluoroborate: yellow needles from methanol, Mp. 213-215°C (decomposes)
Diperchlorate: yellow crystals from methanol, Mp. 207-210°C (decomposes)
Dinitrate: yellow crystals from propanol-ether, Mp. 213-215°C (decomposes)
Ditosylate: light yellow powder from ethanol-ether, Mp. 222-224°C (decomposes)

Example 3

Determination of activity of lactate dehydrogenase in serum

2.5 mL of a sodium pyrophosphate buffer (pH 8.6), containing 48 mmol L-lactate is mixed with 0.2 mL of a solution, containing 0.6-1.2 mg NAD⊕ per mL of a dilute buffer solution (potassium phosphate, pH 7.4) and at least 0.2 mg diaphorase per mL. 0.2 mL of an aqueous solution, containing at least 1 mg/mL 2-benzothiazolyl-(2)-3-phenyl-5-(4-N-trimethylammoniumphenyl)tetrazolium-dichloride is added to this mixture. The reaction is set in motion by addition of 0.2 mL serum. The change in extinction per minute (ΔE/min) in the first 5 minutes after starting is measured at 25°C versus a blind value (546 nm) and the volume activity determined according to the following formula:

$$\text{Volume activity (U/L)} = \frac{\Delta E/\text{min} \cdot V \cdot 1000}{\varepsilon \cdot d \cdot v}$$

$\varepsilon = 27.4$ ($cm^2/\mu mol$), d = layer thickness (cm), v = sample volume, V = test volume; 1000 = conversion factor from mL to L.

Similarly, the activities of the following enzymes can be determined:

a.) α-hydroxybutyric acid dehydrogenase with α-hydroxybutyric acid
b.) Malate dehydrogenase, with malate
c.) Sorbitol dehydrogenase, with D-sorbitol
d.) Alcohol dehydrogenase, with ethanol.

Example 4

Determination of glycerol in serum

2.3 mL buffer (0.2 mol glycine/sodium carbonate buffer, pH = 8.0 - 9.0) is mixed with 0.2 mL of a solution, containing 25 mg ATP, 0.5 mg glycerokinase and 1.0 mg α-glycerophosphate dehydrogenase. 0.2 mL of solution, containing 0.6-1.2 mg NAD⊕ per mL of a dilute buffer solution (potassium phosphate, pH 7.4) and at least 0.2 mg diaphorase per mL is added to this mixture. 0.2 mL of an aqueous solution is then added, containing at least 1 mg/mL 2-benzothiazolyl-(2)-3-phenyl-5-(4-N-trimethylammoniumphenyl)tetrazolium-dichloride. The reaction is set in motion by adding 0.1 mL serum.

Immediately after serum addition, the initial extinction E_1 is interpreted at 546 nm; after 25 minutes, the final extinction E_2 is determined and from the difference ΔE (= E_2-E_1), the content is calculated according to the following formula:

$$\text{Concentration } [\mu g/mL] = \frac{\Delta E \cdot V \cdot MW}{\varepsilon \cdot d \cdot v}$$

V = test volume
v = sample volume
MW = molecular weight
ε = 27.4 (cm^2/mol)
d = layer thickness (cm)

The following substrates, important in clinical chemistry, can be determined similarly:

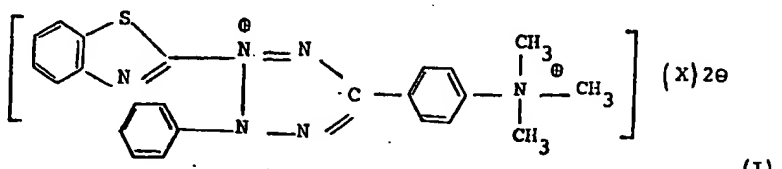
Glucose with ATP, hexokinase and NADP, glucose-6-phosphate dehydrogenase
α-hydroxybutyric acid with α-hydroxybutyric acid dehydrogenase
Malic acid with malate dehydrogenase
Neutral fats (after saponification with ethanolic KOH and determination of glycerol content)

Ethyl alcohol with alcohol dehydrogenase

D-sorbitol with sorbitol dehydrogenase

Claims

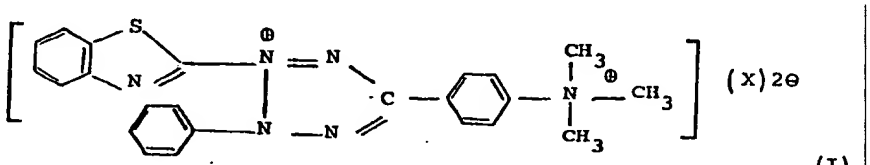
1.) 2-benzothiazolyl-(2)-3-phenyl-5-(4-N-trimethylammonium-phenyl)tetrazolium salts of formula I



(I),

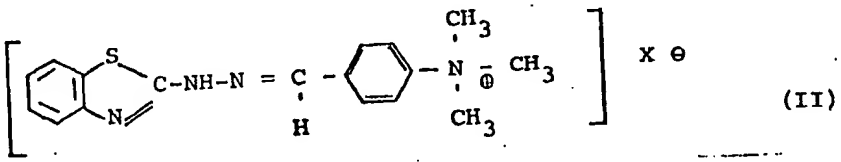
in which X denotes one or more anions of organic or inorganic acids.

2.) Method for preparation of benzothiazolyl-(2)-3-phenyl-5-(4-N-trimethylammonium-phenyl)tetrazolium salts of formula (I)



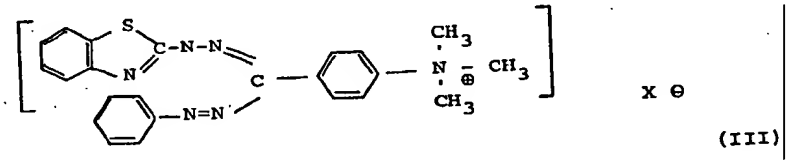
(I),

in which X denotes one or more anions of organic or inorganic acids,
characterized by the fact that a hydrazone of formula (II)



(II)

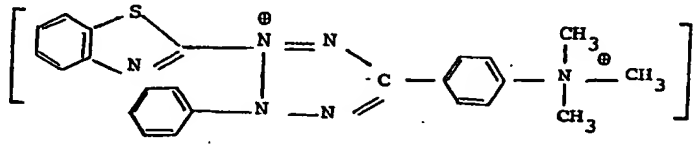
in which X⊖ has the aforementioned meaning,
is converted in known fashion with a reactive benzenediazonium salt and the obtained formazan of formula III



(III)

in which X⊖ has the aforementioned meaning,
is converted with an appropriate oxidation agent and, if desired, the anion X⊖ modified with corresponding alkali salts or correspondingly loaded ion exchangers.

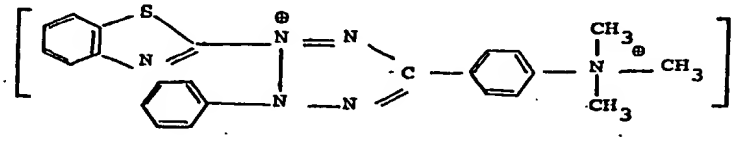
3.) Use of 2-benzothiazolyl-(2)-3-phenyl-5-(4-N-trimethylammonium-phenyl)tetrazolium salts of formula I



(I),

in which X denotes one or more anions of organic or inorganic acids,
for detection of biological reduction processes.

4.) Test reagent for detection of reduction processes, characterized by a content of 2-benzothiazolyl-(2)-3-phenyl-5-(4-N-trimethylammonium-phenyl)tetrazolium salts of formula I



(I),

in which X denotes one or more anions of organic or inorganic acids.